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Methoxy-Substituted Hydroxychalcone Reduces Biofilm Production, Adhesion and Surface Motility of *Acinetobacter baumannii* by Inhibiting *ompA* Gene Expression

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An increasing lack of available therapeutic options against *Acinetobacter baumannii* urged researchers to seek alternative ways to fight this extremely resistant nosocomial pathogen. Targeting its virulence appears to be a promising strategy, as it offers considerably reduced selection of resistant mutants. In this study, we tested antibiofilm potential of four synthetic chalcone derivatives against *A. baumannii*. Compound that showed the greatest activity was selected for further evaluation of its antivirulence properties. Real-Time PCR was used to evaluate mRNA expression of biofilm-associated virulence factor genes (*ompA*, *bap*, *abal*) in treated *A. baumannii* strains. Also, we examined virulence properties related to the expression of these genes, such as fibronectin- and collagen-mediated adhesion, surface motility, and quorum-sensing activity. The results revealed that the expression of all tested genes is downregulated together with the reduction of adhesion and motility. The conclusion is that 2-methoxy-2'-hydroxychalcone exhibits antivirulence activity against *A. baumannii* by inhibiting the expression of *ompA* and *bap* genes, which is reflected in reduced biofilm formation, adhesion, and surface motility.

Keywords: *Acinetobacter baumannii* • chalcones • virulence factors • polymerase chain reaction • gene expression

Introduction

Acinetobacter baumannii is recognized as one of the most troublesome nosocomial pathogens, due to its outstanding ability to rapidly develop antimicrobial resistance and to persist in wide range of environmental conditions.^[1] Extreme tolerance to desiccation and formation of highly recalcitrant biofilms provide *A. baumannii* potential to survive for extended periods on hospital surfaces, materials, and medical devices, thus promoting hospital outbreaks and epidemics.^[2] The ever-increasing number of extensively drug-resistant (XDR) isolates, resistant to carbapenems, as well as emergence of pandrug-resistant (PDR) strains, emphasizes the indisputable need for discovery and development of new therapeutic strategies.^[3] One such strategy that is being investigated deals with targeting of the *A. baumannii* virulence factors, which provides possibility to disarm pathogens, while minimally affecting their growth, thereby generating much weaker selection of resistant mutants.^{[4][5]}

Besides the well-known resistance to desiccation and an ability of biofilm formation that mediate the so-called "persist and resist" strategy of virulence, there are now numerous recognized virulence factors in *A. baumannii*.^[6] These include adherence mechanisms, motility, siderophore-mediated iron acquisition systems, activities of polysaccharide membrane and outer membrane protein phospholipases, alteration in penicillin-binding proteins (PBPs), outer membrane vesicles (OMVs), and mechanisms of immune evasion.^[7] Of the particular interest is outer membrane protein A (OmpA), the most abundant protein in outer membrane of *A. baumannii*, with well-characterized virulence involvement.^[8] OmpA promotes bacterial adherence and invasion, as well as subsequent apoptosis of host epithelial cells, partially by acting as a major fibronectin binding ligand.^[9-11] Additionally, this protein induces the biogenesis of cytotoxic OMVs, thus further enhancing host cell apoptosis.^{[12][13]} Also, OmpA contributes to the resistance, survival, and persistence of *A. baumannii*, by promoting immune evasion, biofilm formation, surface motility, and multidrug resistance.^{[8][14-17]} Biofilm-associated protein (Bap) is another large outer membrane protein that enables formation of fully mature biofilms and increases adherence to human cells,^{[18][19]} whose inhibition was shown to diminish the virulence potential of *A. baumannii*.^[20] Finally, the quorum-sensing (QS) system in *A. baumannii*, consisting of auto-inducer synthase (AbaI), acyl-homoserine lactone (AHL) signal molecules, and AbaR receptor,^[21] was also shown to influence the expression of virulence factors such as biofilm formation^[22] and surface motility.^[16] Moreover, the attenuated virulence in a zebrafish infection model was demonstrated for *abal* deficient mutants.^[23]

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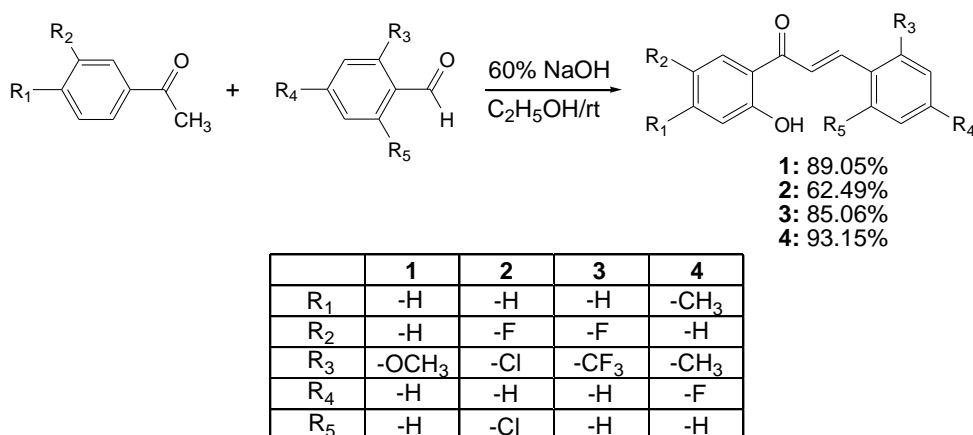
Chalcones are natural compounds with simple common chemical scaffold 1,3-diaryl-2-propen-1-one (chalconoide), which can be found in fruits, vegetables, spices, teas, and other products of some plant species.^[24] A wide variety of biological and pharmacological activities of chalcones have been observed. These include antioxidant properties, anti-inflammatory effects, chemopreventive and cytotoxic activities, anti-hyperglycemic and hypolipidemic activities, cardioprotective and neuroprotective effects, antibacterial, antifungal, antiviral, and antiparasitic activities, and many others.^[25-27] In addition to natural isolation, chalcones can be obtained by chemical synthesis that allows construction of compounds with targeted activity and minimal side effects.^{[25][28]} In that sense, a variety of synthetic chalcones were screened for antimicrobial activity and lots of them showed great potential in this respect.^[29]

In our recent study, we had demonstrated that among the four differently substituted 2'-hydroxychalcones, 2-methoxy substituted derivative is the most active in inhibition of bacterial biofilm production.^[30] For the purpose of this study, we synthesized two differently substituted 2'-hydroxy-5'-fluorochalcones and one 2'-hydroxy-4'-methylchalcone, and tested their antibiofilm potential, along with 2-methoxy-2'-hydroxychalcone from previous study, against *A. baumannii*. Further, since the biofilm production itself is not associated with *A. baumannii* virulence, we selected compound that exhibited greatest antibiofilm activity for examination of its influence on biofilm-related virulence factors. We monitored expression levels of biofilm-associated virulence factor genes, as well as fibronectin- and collagen-mediated adhesion, surface motility, and QS activity of *A. baumannii* strains treated with the selected chalcone compound. Also, we examined crystallinity and thermal properties of this compound to get better insights into its stability, solubility, and bioavailability.

Results

Synthesis

Compounds **1-4** were obtained by base-catalyzed Claisen-Schmidt condensation (Scheme 1) in the form of yellow colored powders. Structures were verified by Fourier-transform infrared (FTIR), ¹H NMR, ¹³C NMR and ¹⁹F NMR spectroscopy techniques, and high-resolution electrospray ionization mass spectrometry (HR-ESI-MS). NMR spectra are provided in Supporting information.



Scheme 1. Synthesis of compounds **1-4**.

Antibiofilm Activity of Compounds **1-4**

Compounds **1-4** were screened for antibiofilm activity against *A. baumannii* reference strain and wound isolate at concentration of 70 µg ml⁻¹ (Supplementary Figure S1). Significant activity against *A. baumannii* ATCC 19606 was exhibited by compounds **1**, **2**, and **4**. However, only compound **1** significantly inhibited production of biofilm in *A. baumannii* wound isolate. Subsequently, we selected compound **1** for further examination of crystallinity, thermal characteristics, and biofilm-associated virulence factor genes expression. Also, we evaluated its biofilm inhibitory activity at 35 µg ml⁻¹ and 10 µg ml⁻¹, and revealed dose-dependent influence (Figure 3A). Whereas, concentration of 35 µg ml⁻¹ still yielded significant biofilm inhibition in both strains, concentration of 10 µg ml⁻¹ had little effect.

Crystallinity and Thermal Properties of Compound **1**

To further examine the structure and properties of compound **1**, we employed X-ray diffraction (XRD), microscopy, and thermal analyses. Based on the numerous sharp peaks, observed from the XRD pattern (Figure 1B), the sample possesses a highly crystalline nature which is also confirmed by optical microscopy (Figure 1A). The most intense reflections of the incident beam were detected at the following 2θ angles: 26.25 and 24.85 along with a triplet in the interval 14.05-15.4. Regarding the investigation of thermal stability, based on the data obtained from thermogravimetric differential thermal analysis (TGA/DTA; Figure 1C), it can be concluded that the sample is stable up to 250 °C, after which it goes through the rapid weight loss of about 90%. The combustion of remaining decomposition products took place at around 490 °C, which could be also noticed as a big exothermic peak on the DTA signal.

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Differential scanning calorimetry (DSC) further revealed that two endothermic events took place (Figure 1D). The first one has a substantially lower intensity and the second one clearly represents the melting of the sample with a detected melting temperature of 101.4 °C. The first peak occurs around 66 °C and it could be attributed to the removal of adsorbed moisture. However, the TGA signal did not record any weight loss in this region, thus it is possible that this peak corresponds to some structural changes and arrangement of molecular conformation. The molecular conformation of chalcone is influenced by the intramolecular forces, and based on the composition of the sample presented here, results obtained from FTIR spectroscopy, and XRD, there is more than one conformation present in the sample.

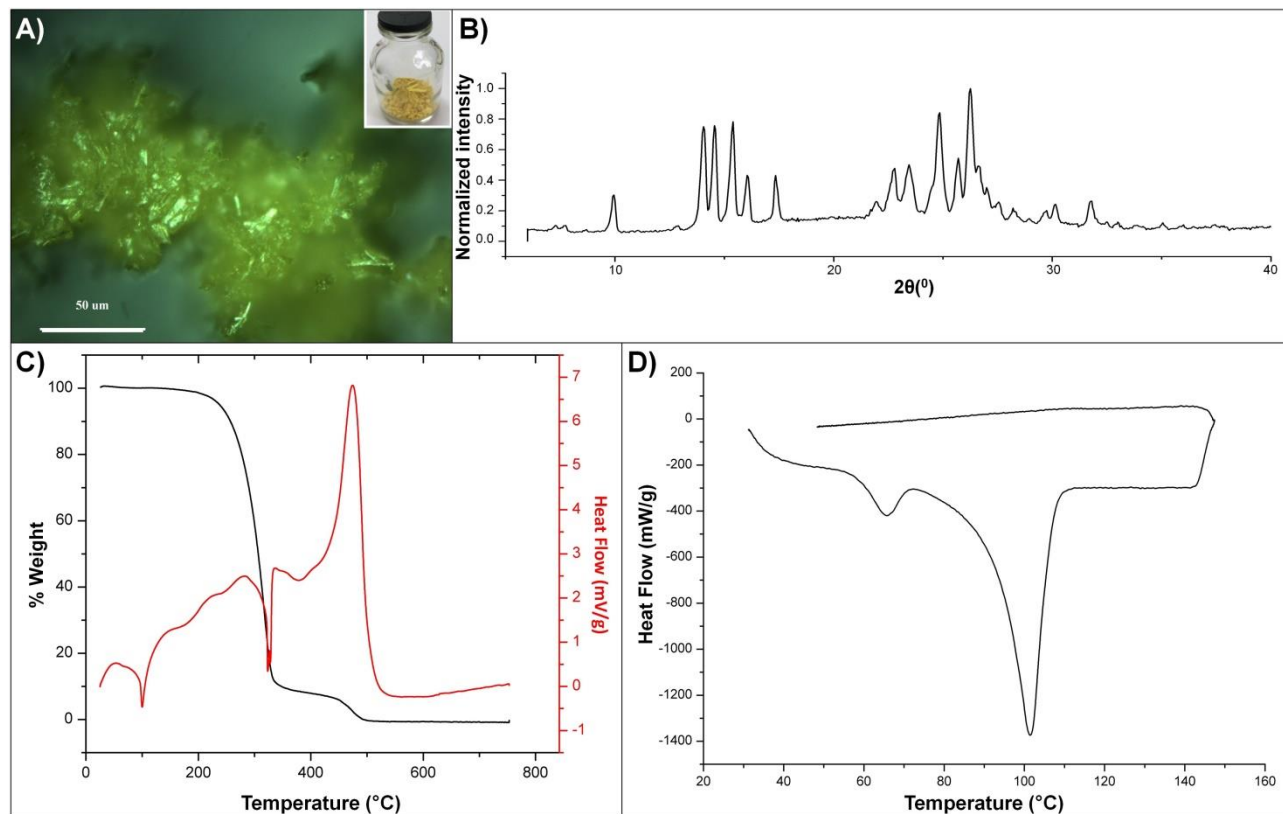


Figure 1. Crystallinity and thermal properties of compound **1**. (A) Microscopic and macroscopic evaluation, (B) XRD, (C) TGA/DTA, (D) DSC.

The Expression of *ompA*, *bap*, and *abaI* Genes is Downregulated by Compound **1**

The mRNA expression, influenced by compound **1** at decreasing sub-minimum inhibitory concentrations (sub-MICs) from 70 to 10 µg ml⁻¹, was investigated for several biofilm-associated virulence factor genes of *A. baumannii* (Figure 2). Remarkably, all the tested genes exhibited significant downregulation of the expression by all tested concentrations. The mRNA level of *ompA*, which encodes a protein involved in numerous virulence-associated traits of *A. baumannii*, was dose-dependently decreased in both tested strains. Most notably, 1.58-fold and 1.85-fold downregulation was achieved at concentration of 70 µg ml⁻¹ in strains ATCC 19606 and 766, respectively. Interestingly, the reduction of mRNA level of *Bap*-encoding gene was greater when using lower concentrations. In particular, its expression was almost twofold reduced in *A. baumannii* ATCC 19606 by compound **1** at 10 µg ml⁻¹. Finally, mRNA expression of *abaI*, a gene that encodes auto-inducer synthase, an essential component of *A. baumannii* QS system, was decreased in *A. baumannii* ATCC 19606, but also in a reverse dose-dependent manner. The expression of this gene was not detected in strain 766.

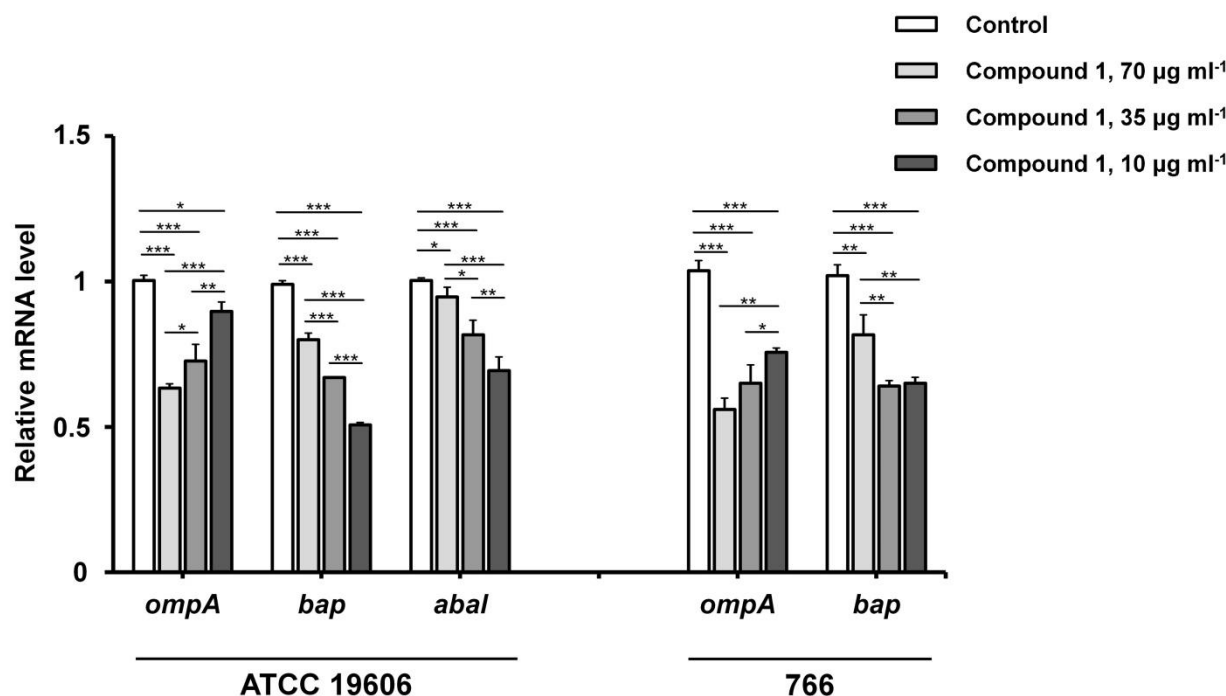


Figure 2. The mRNA expression of *ompA*, *bap*, and *abal* genes in *A. baumannii* treated with different concentrations of compound **1**. Data are presented as mean values of three experiments (\pm SD). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to the control group.

Compound **1** Inhibits Fibronectin- and Collagen-Mediated Binding of *A. baumannii*

The adherence ability of *A. baumannii* strains treated with compound **1** was evaluated through fibronectin- and collagen-binding affinity. *A. baumannii* ATCC 19606 exhibited much higher binding affinity to both of these extracellular matrix (ECM) proteins, in comparison to the wound isolate (Figures 3B and 3C). Strikingly, the cells of the standard strain were almost completely deprived of the binding ability to both fibronectin and collagen, when incubated in the presence of compound **1** at $70 \mu\text{g ml}^{-1}$. The binding affinity of this strain was substantially reduced by compound **1** at other two tested concentrations as well. In case of *A. baumannii* 766, however, only the collagen-binding affinity was significantly reduced by compound **1** at all tested sub-MICs.

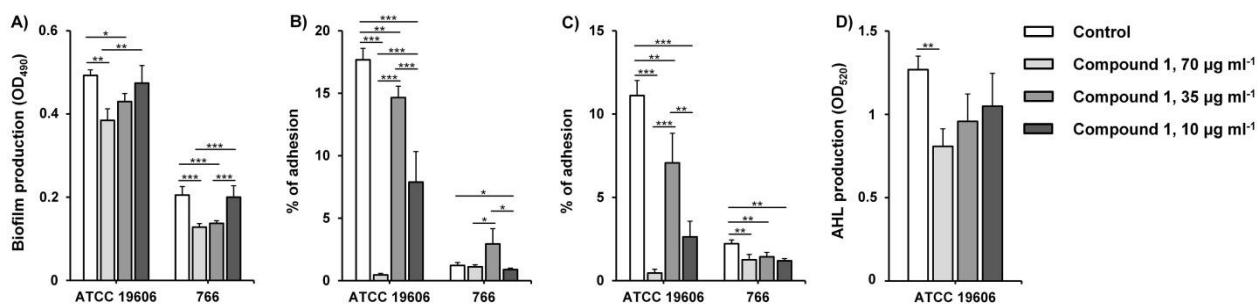


Figure 3. Comparisons between control and treated groups of *A. baumannii* ATCC 19606 and *A. baumannii* 766. (A) Biofilm production, represented as OD values of the extracted safranin dye at 490 nm, (B) Fibronectin- and (C) Collagen-binding ability represented as the percentage of bound bacterial cells, (D) AHL production, measured as OD values of dark brown colored ferric hydroxamate complexes at 520 nm. Data are presented as mean values of three experiments (\pm SD). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to the control group.

Surface Motility of *A. baumannii* Wound Isolate is Inhibited by Compound **1**

Surface motility was not exhibited by *A. baumannii* ATCC 19606, thus only *A. baumannii* 766 was used for the evaluation of antimotility activity of compound **1**. The surface motility of *A. baumannii* 766 was considerably inhibited by compound **1** at $70 \mu\text{g ml}^{-1}$ and $35 \mu\text{g ml}^{-1}$, while the lowest tested concentration had no effect, since the surface of entire plate was covered as in the case of untreated control (Figure 4). Comparison of migration areas revealed that the treatment with $70 \mu\text{g ml}^{-1}$ resulted in 150.16 mm^2 of covered surface area, which is less compared to the extent of migration exhibited by bacteria treated with $35 \mu\text{g ml}^{-1}$ (229.94 mm^2), indicating the existence of dose-dependent activity.

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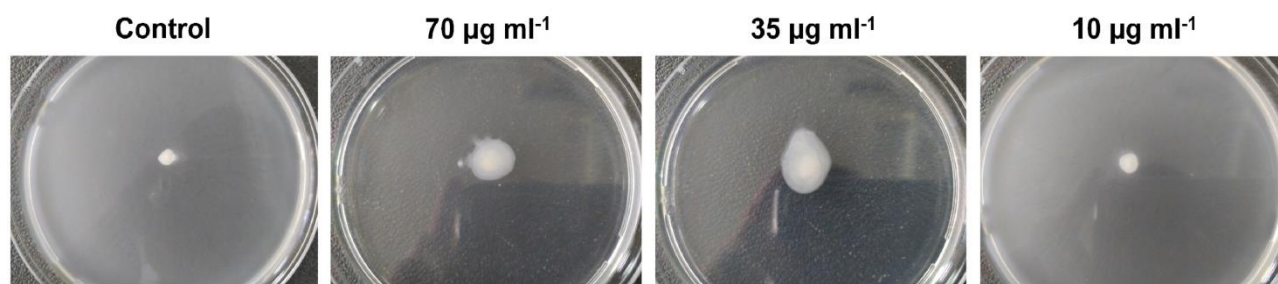


Figure 4. Surface motility of *A. baumannii* 766 treated with decreasing sub-MICs of compound **1** compared to untreated control.

Compound **1** Reduces AHL Secretion in *A. baumannii* ATCC 19606

Since the expression of Abal-encoding gene was not detected in *A. baumannii* wound isolate, we evaluated the influence of compound **1** on AHL production only in strain ATCC 19606. The results revealed that the production is reduced by compound **1**, at all tested concentrations, in a dose-dependent manner (Figure 3D). However, significant reduction was achieved only with 70 µg ml⁻¹, which inhibited AHL secretion by 36.22%.

A. baumannii Growth is not Affected by Tested Sub-MICs of Compound **1**

In order to demonstrate that investigated sub-MICs (70 µg ml⁻¹, 35 µg ml⁻¹, and 10 µg ml⁻¹) of compound **1** do not affect *A. baumannii* growth, we measured bacterial cell growth at five different time points. According to constructed growth curves (Supplementary Figure S2), it can be concluded that tested concentrations do not inhibit *A. baumannii* growth. Subsequently, it is reasonable to consider that potential inhibition of virulence by compound **1** will not enhance selection of resistant mutants, i.e. compound **1** at selected concentrations is an appropriate antivirulence drug candidate.

Discussion

So far, myriad of chalcone structures have displayed efficient antibacterial activities against both Gram-positive and Gram-negative species (aerobic or anaerobic), *Mycobacterium tuberculosis*, and other resilient genera.^{[29][31]} Besides the substantially efficient growth inhibition activities, with MICs reaching the values below 1 µg ml⁻¹ in some cases,^{[32][33]} several antivirulence properties of chalcones have also been documented, including the inhibition of biofilm formation, glycocalyx production, motility, and adhesion,^{[30][34][35]} the quorum-quenching activity,^[36] inhibition of certain genes' expression and bacterial toxin production,^[37] and inhibition of the efflux pumps.^[38-40]

Biofilm formation in *A. baumannii* is responsible for colonization of abiotic and biotic surfaces, including medical devices and host tissues, thus enabling the persistence and spreading of infection in hospital settings.^{[6][35]} Besides, increased resistance or tolerance of biofilm-associated *A. baumannii* cells to antimicrobial drugs, including carbapenems, aminoglycosides, and colistin, has been documented in several studies.^[41-43] Also, *A. baumannii* biofilm formation was shown to contribute to the immune evasion,^[44] and could be related to several other virulence properties, perhaps at the level of gene expression. Earlier, we demonstrated that a synthetic 2-methoxy substituted 2'-hydroxychalcone has the greatest antibiofilm activity in comparison to other tested variously substituted 2'-hydroxychalcones.^[30] For the purpose of this study we synthesized three new chalcones (compounds **2-4**) and compared their antibiofilm activity to that of 2-methoxy-2'-hydroxychalcone (compound **1**). One of the newly-synthesized compounds was derivative of 2'-hydroxy-4'-methylchalcone, while the remaining two were derivatives of 2'-hydroxy-5'-fluorochalcone. Still, the best activity was exhibited by 2-methoxy-2'-hydroxychalcone, possibly due to the presence of methoxy group, considering that potentiating effect of methoxy group on antibiofilm activity is known from some previous studies.^{[45][46]} We therefore selected this compound for further investigation of its antivirulence properties. To demonstrate that this chalcone is not just a potent antibiofilm agent, we tested its influence on several important biofilm-associated virulence factor genes of *A. baumannii*. All of the tested biofilm-associated genes exhibited significant downregulation, however, the most notable finding is significant inhibition of *ompA* gene expression. As described previously, OmpA is a protein which can induce adherence and invasion of bacteria to human epithelial cells, and trigger host cell apoptosis, promote antimicrobial resistance, inhibit the alternative complement pathway, and induce the biogenesis of cytotoxic OMVs, biofilm formation, and surface motility.^{[1][8]} The combination of these features results in a strain that is much more virulent and associated with higher morbidity and mortality rates in infected patients. Recent study has demonstrated that bacteremic or non-bacteremic pneumonia-causing strains express much higher levels of *ompA* compared to colonizing non-pathogenic strains. Multivariate analysis, performed within latter study, further showed that *ompA* expression is an independent risk factor for pneumonia, bacteremia, and mortality in *A. baumannii* infected patients. Additionally, the same study demonstrated a substantial role of OmpA in mortality and dissemination of infection in a murine peritoneal sepsis model.^[47]

In order to demonstrate the potential *ompA*-dependent antivirulence activity of compound **1**, we decided to further evaluate phenotypic features associated with this gene, such as adhesion and motility. ECM proteins are important mediators in bacterial adhesion to host tissues, and *A. baumannii*

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displays high affinity binding to these proteins, including fibronectin and collagen.^[48] Furthermore, it was shown that OmpA plays a significant role in fibronectin-mediated adhesion.^[31] Therefore, we tested fibronectin- and collagen-mediated binding affinity of *A. baumannii*, and found that it is considerably reduced by compound **1**. Only the fibronectin binding of *A. baumannii* wound isolate was not significantly affected, most likely due to generally weaker ECM-binding affinity exhibited by this strain. Fibronectin-associated antiadherence properties of similar chalcone compounds were previously demonstrated against methicillin-resistant *Staphylococcus aureus* (MRSA).^[34] Also, comparable results were reported for AOA-2, a cyclic hexapeptide that was shown to inhibit the adhesion of *A. baumannii* strain ATCC 17978 to A549 cells. This peptide, also a potent biofilm inhibitor, was further tested *in vivo*, in a peritoneal sepsis murine model, where it succeeded to significantly reduce spleen and lung bacterial loads, and to decrease mortality. Most importantly, the authors have demonstrated that AOA-2 interact with OmpA protein.^[49]

Two independent forms of motility have been described in *A. baumannii*: twitching and surface motility.^[6] Although, the role of motility in virulence of *A. baumannii* is still not well established, there are some observations that indicate its potential contribution to the increased virulence. For example, a hypermotile variant of *A. baumannii* ATCC 17978 strain with disrupted *hns*-like gene displayed increased virulence potential in the *Caenorhabditis elegans* infection model.^[50] Similarly, several isolated rifampin-resistant *A. baumannii* ATCC 17978 *rpoB* mutants showed defective surface motility and attenuated phenotype in *C. elegans* fertility model.^[51] According to Clemmer *et al.*,^[16] the expression of *ompA* is linked to the surface motility of *A. baumannii*, therefore we tested the impact of compound **1** on this type of motility. Unluckily, *A. baumannii* ATCC 19606 is a non-motile strain,^[52] so it could not be used for evaluation of antimotility activity. However, surface motility of *A. baumannii* wound isolate was considerably inhibited by compound **1**. The QS system is also involved in surface motility of *A. baumannii*, but since the expression of *abal* was not detected in wound isolate, it could be assumed that impaired motility is related to the inhibition of *ompA* expression. Interestingly, two recent studies showed that honokiol, magnolol, and curcumin, which are plant products same as chalcones, also co-inhibit biofilm formation and surface motility in *A. baumannii* ATCC 17978 strain. These studies further demonstrated increased survival of *C. elegans* nematodes infected with treated strains in comparison to positive controls. Consequently, these results provide additional evidence that impaired biofilm formation and surface motility in *A. baumannii* strains result in reduced virulence *in vivo*.^{[53][54]}

Finally, since we demonstrated significant downregulation of *abal* in *A. baumannii* ATCC 19606, we decided to test the production of AHLs, signal molecules that are synthesized by Abal, as a part of QS system.^[21] *N*-(3-hydroxydodecanoyl)-L-homoserine lactone is so far the only described AHL molecule in *A. baumannii*, whose binding to AbaR was shown to contribute to biofilm forming ability and surface motility through the modification of gene expression. More importantly, some authors have documented its association with virulence.^{[23][55]} Regarding the quorum-quenching ability of chalcone-related compounds in *A. baumannii*, i.e. the ability to suppress QS-mediated intercellular communication, Bhargava *et al.* discovered potent activity of certain *Glycyrrhiza glabra* flavonoids.^[55] This finding is in agreement with potent quorum-quenching activity of compound **1** against *A. baumannii* ATCC 19606, that we observed in this study, however, we could not test its impact on QS system of *A. baumannii* 766, since the expression of key QS element Abal, was not detected by qPCR.

Conclusions

Thus far, we showed that the use of 2-methoxy, 2'-hydroxy substituted chalcone could provide us with a possibility to reduce two key features that make *A. baumannii* one of the most problematic pathogens of today, that is, ability to persist and to rapidly develop antimicrobial resistance. By inhibiting its biofilm production, this compound makes these bacteria vulnerable to a range of exterior agents, whereas attenuation of its virulence substantially reduces pathogenic infections and severe outcomes without direct activity against its growth, consequently generating much slower selection of resistant mutants. The fact that the impact of chalcones or related compounds on the expression of *A. baumannii* key virulence factors have not been studied earlier, further contributes to the significance of this study. Having described structure and properties of this potential antivirulence agent, we provide information on its stability, solubility, and bioavailability that could be useful in future investigations, perhaps design of drug formulations for *in vivo* studies.

Experimental Section

Materials and Methods

Reagents purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA) were used for synthesis of compounds. Also, 96% (v/v) ethanol, NaOH, HCl, dimethyl sulfoxide (DMSO), type I recombinant human collagen, Triton X-100, ethyl acetate, and hydroxylamine were purchased from Sigma-Aldrich Inc. Tryptic soy broth (TSB), agarose, and tryptone were bought from Torlak (Belgrade, Serbia), whereas Mueller-Hinton broth (MHB) and 0.5% safranin were procured from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Also, human fibronectin, purchased from Serva (Heidelberg, Germany) was used in this study. Thin-layer chromatography (TLC) was conducted with silica gel 60 F₂₅₄ aluminum sheets (Merck, Darmstadt, Germany). FTIR spectra were recorded on spectrometer Nicolet iS10 (Thermo Fisher Scientific, Waltham, MA, USA), using attenuated total reflectance (ATR) mode. Measurements

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were performed in a spectral range of 400–4000 cm^{-1} with a resolution of 4 cm^{-1} and the number of scans was 32. NMR spectra were taken on Bruker 400 spectrometer (Bruker, Billerica, MA, USA), at 400, 100 and 376.46 MHz for ^1H , ^{13}C and ^{19}F NMR spectra, respectively, using tetramethylsilane as an internal standard in deuterated chloroform (CDCl_3). The chemical shifts were expressed in ppm and the coupling constants (J) in Hz. Splitting patterns were designated as singlet (s), doublet (d), triplet (t) and multiplet (m). High-resolution mass spectra were measured by Agilent 6210 Time-of-Flight LC/MS system (Agilent Technologies, Palo Alto, CA, USA). The microscopy analysis was performed using the OPTICA B-500MET light microscope (Optica SRL, Ponteranica, Italy). Image was collected in reflective mode with OPTIKAM PRO 8LT - 4083.18 camera equipped with scientific-grade CCD sensor. XRD from sample powder was obtained on Philips PW 1050 diffractometer (Philips, Eindhoven, The Netherlands) using $\text{Cu-K}\alpha$ radiation (Ni filter). The sample was scanned in the 2θ range of 10° to 70° , with a scanning step width of 0.05° , and speed of 2 s per step. Thermal analysis was conducted on SETARAM apparatuses (Caluire-et-Cuire, France), SETSYS evolution TGA/DTA and DSC 131 EVO controlled by CALISTO software. For TGA/DTA sample was placed in an alumina crucible and heated from room temperature up to 900°C with a heating rate of $10^\circ\text{C min}^{-1}$. The phase transition within the region of thermal stability was evaluated by DSC. For this purpose accurately weighed sample was hermetically sealed in 30 μl aluminum pans and heated from 30°C to 150°C at a rate of $10^\circ\text{C min}^{-1}$ in nitrogen gas flow. EZ Read 400 Microplate Reader (Biochrom, Holliston, MA, USA) was used for the measurement of optical density (OD) values.

General Procedure for the Synthesis of Compounds 1-4

Compounds were synthesized by Claisen-Schmidt condensation of non-substituted (**1**) or substituted 2-hydroxyacetophenone (**2-4**) and substituted benzaldehyde, in the presence of relatively strong base (60% (w/v) NaOH), at room temperature. 2-hydroxyacetophenone (0.012 mol) and benzaldehyde (0.01 mol) were simultaneously dissolved in 96% (v/v) ethanol (10 ml) with continuous stirring. Resulting colorless solution was supplemented gradually with 60% (w/v) NaOH in small portions (20 g in total), until the blood-red color was achieved. The solution was then stirred overnight at room temperature, in order to precipitate the chalcone as orange sodium salt and the resulting mixture was kept at 0°C for 24 h. Afterwards, the mixture was diluted with ice water and acidified with cold 1 mol l^{-1} HCl, gradually until the pH of approx. 3 was reached. The resulting yellow precipitate was then filtered in vacuum, washed with ice water to a near neutral pH level, and the crude mixture was left to air-dry in dark, after which it was purified by preparative TLC using the silica gel plates and toluene as eluent. After removal of toluene under vacuum, the crude products recrystallized from ethanol.

(E)-1-(2-hydroxy-5-fluorophenyl)-3-(2,6-dichlorophenyl)-prop-2-en-1-one (**2**)

Yellow crystals, Yield: 62.49%. IR (ATR): 1645.1, 1578.9, 1478.9, 1355.4, 1240.9, 1170.0, 972.3, 850.5, 835.5, 784.1, 722.1, 677.2. ^1H NMR (400 MHz, CDCl_3): 12.43 (s, -OH, 1H); 8.02 (d, $J=16$, a, 1H); 7.75 (d, $J=16$, b, 1H); 7.51-7.49 (m, ArH-C(6'), 1H); 7.42 (d, $J=8$, ArH-C(3), ArH-C(5), 2H); 7.26-7.22 (m, ArH-C(4), ArH-C(4'), 2H); 7.03-6.99 (m, ArH-C(3'), 1H). ^{13}C NMR (100 MHz, CDCl_3): 192.90; 159.88; 156.13; 153.76; 139.18; 135.44; 132.04; 130.34; 129.05; 128.34; 124.46; 124.22; 120.00; 119.92; 119.43; 119.37; 114.92; 114.69. ^{19}F NMR (376.46 MHz, CDCl_3): $\delta = -123.78$. HRMS (ESI) m/z calcd for $\text{C}_{15}\text{H}_9\text{Cl}_2\text{FO}_2$ [M] $^+$ 311.135 found 311.839.

(E)-1-(2-hydroxy-5-fluorophenyl)-3-(2-trifluoromethylphenyl)-prop-2-en-1-one (**3**)

Yellow crystals, Yield: 85.06%. IR (ATR): 1644.3, 1586.8, 1574.6, 1484.1, 1351.8, 1286.4, 1238.9, 1099.8, 1061.1, 1034.7, 1015.6, 970.9, 850.8, 771.4, 757.5, 719.2, 680.3, 651.1. ^1H NMR (400 MHz, CDCl_3): 12.50 (s, -OH, 1H); 8.31 (d, $J=15.20$, a, 1H); 7.85 (d, $J=7.6$, ArH-C(6'), 1H); 7.69 (d, $J=7.6$, ArH-C(3), 1H); 7.66-7.62 (m, ArH-C(6), 1H); 7.56-7.53 (m, ArH-C(4), ArH-C(5), 2H); 7.49 (d, $J=15.20$, b, 1H); 7.28-7.23 (m, ArH-C(4'), 1H); 7.03-6.99 (m, ArH-C(3'), 1H). ^{13}C NMR (100 MHz, CDCl_3): 192.47; 159.92; 156.11; 153.74; 141.57; 133.48; 132.22; 130.27; 128.10; 126.51; 126.45; 124.42; 124.19; 123.99; 120.11; 114.74. ^{19}F NMR (376.46 MHz, CDCl_3): $\delta = -124.00$ (-F); $\delta = -58.78$ (-CF₃). HRMS (ESI) m/z calcd for $\text{C}_{16}\text{H}_{10}\text{F}_4\text{O}_2$ [M] $^+$ 310.243 found 311.084.

(E)-1-(2-hydroxy-4-methylphenyl)-3-(2-methyl-4-fluorophenyl)-prop-2-en-1-one (**4**)

Yellow crystals, Yield: 93.15%. IR (ATR): 3015.3, 1640.7, 1573.2, 1504.2, 1493.9, 1365.5, 1286.9, 1234.9, 1208.3, 1167.9, 1146.06, 975.9, 955.7, 943.8, 849.7, 784.2, 735.1. ^1H NMR (400 MHz, CDCl_3): 12.79 (s, -OH, 1H); 8.15 (d, $J=15.6$, a, 1H); 7.79 (d, $J=8.4$, ArH-C(6'), 1H); 7.71-7.67 (m, ArH-C(6), 1H); 7.51 (d, $J=15.2$, b, 1H); 6.96-6.93 (m, ArH-C(5), ArH-C(5'), 2H); 6.84 (s, ArH-C(3), 1H); 6.76-6.74 (m, ArH-C(3'), 1H); 2.49 (s, -C(2)-CH₃, 3H). ^{13}C NMR (100 MHz, CDCl_3): 193.01; 163.17; 163.88; 162.67; 148.17; 141.37; 141.32; 141.24; 130.05; 129.52; 128.61; 128.52; 121.15; 120.21; 118.75; 117.81; 117.60; 113.74; 113.53; 22.00; 19.95. ^{19}F NMR (376.46 MHz, CDCl_3): $\delta = -109.90$. HRMS (ESI) m/z calcd for $\text{C}_{17}\text{H}_{15}\text{FO}_2$ [M] $^+$ 270.298 found 270.829.

Spectral data of previously published compound **1**^[30] are provided in Supporting information.

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Bacterial Strains and Growth Conditions

A. baumannii wound isolate (766) obtained from Clinical Medical Center Zvezdara Belgrade and *A. baumannii* type strain ATCC 19606 (KWIK-STIK™, Microbiologics Inc., St. Cloud, MN, USA) were used in this study. The wound isolate (766) was initially identified as member of *Acinetobacter calcoaceticus-baumannii* (Acb) complex by VITEK® 2 system (bioMérieux, Craponne, France). The strain was later confirmed as *A. baumannii* on the basis of growth at 44 °C and obtained FTIR spectrum. According to susceptibility testing performed by broth microdilution method and VITEK® 2 system, *A. baumannii* 766 was classified as XDR, susceptible only to colistin. Both strains were grown in TSB at 37 °C for 24 h prior to experiments.

Growth of *A. baumannii* strains in MHB supplemented with compound **1** at concentrations of 70 µg ml⁻¹, 35 µg ml⁻¹, and 10 µg ml⁻¹ was monitored at five different time points (1 h, 3 h, 6 h, 24 h, and 48 h).^[56] Cultures were inoculated at an initial optical density at 600 nm (OD₆₀₀) of 0.04 and incubated at 37 °C. The growth was followed by determining OD₆₀₀ values at each time point.

Biofilm Assay

The biofilm production of *A. baumannii* was tested in the presence of compounds **1-4** at sub-MIC of 70 µg ml⁻¹. Compound that exhibited greatest activity was further evaluated at concentrations of 35 µg ml⁻¹ and 10 µg ml⁻¹. The samples were prepared by using DMSO at concentrations below 1% (v/v) for dissolution. TSB supplemented with an additional 1% glucose was used as the growth medium. Briefly, strains, inoculated at approx. 10⁶ CFU ml⁻¹, were grown in 96-well plates (Sarstedt, Newton, NC, USA) at 37 °C for 24 h. Afterwards, the plates were washed three times with phosphate-buffered saline (PBS) and then fixated by air-drying at 60 °C for 1 h. Fixed biofilms were then stained with 0.5% safranin for 15 min. Unbound dye was rinsed off under running tap water, and the bound dye extracted with 96% (v/v) ethanol. Finally, ODs were measured at 490 nm.^[57]

Quantitative Real-Time PCR

Cultures of *A. baumannii* in MHB, inoculated at approx. 10⁸ CFU ml⁻¹ and treated with 70 µg ml⁻¹, 35 µg ml⁻¹, and 10 µg ml⁻¹ of compound **1**, along with untreated counterparts, were incubated at 37 °C overnight. Then, the total RNA extraction was performed using RNeasy Mini Kit (Qiagen, Hilden, Germany) with a modified lysis step.^[58] DNase I treatment was performed by an Ambion DNasefree™ Kit (Thermo Fisher Scientific, Cambridge, MA, USA). Reversed transcription was done using isolated RNA (1 µg) as a template, according to the manufacturer's protocol (Thermo Scientific, Vilnius, Lithuania). Random hexamers (Applied Biosystems, Foster City, CA, USA) and RiboLock RNase inhibitor (Thermo Scientific, Vilnius, Lithuania) were used in the reactions. Quantification of gene transcripts was performed using KAPA SYBR Fast qPCR Kit (KAPA Biosystems, Wilmington, MA, USA) in 7500 Real-Time PCR 265 system (Applied Biosystems, Foster City, CA, USA) under the following conditions: 3 min at 95 °C activation, 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Normalization was done against the *rpoB* gene using the 2^{-ΔΔCt} method.^[59] Primers used in the study are listed in Table 1. All primers were purchased from Thermo Scientific.

Table 1. The list of primers used in this study.

Gene	Primer sequence (5'→3')	Reference
<i>rpoB</i>	TCCGCACGTAAAGTAGGAAC	[58]
	ATGCCGCCTGAAAAAGTAAC	
<i>ompA</i>	TCTTGGTGGTCACTTGAAGC	[59]
	ACTCTTGTGTTGTGGAGCA	
<i>bap</i>	AATGCACCGGTACTTGATCC	[60]
	TATTGCCTGCAGGGTCAGTT	
<i>abai</i>	CCGCCTTCTCTAGCAGTCA	[61]
	AAAACCCGCAGCACGTAATAA	

Fibronectin- and Collagen-Binding Assays

The methods were performed as described earlier with some modifications.^{[11][64]} Wells of the sterile 96-well plates were coated with human fibronectin (100 µg ml⁻¹) or type I recombinant human collagen (100 µg ml⁻¹) at 4 °C for 16 h. Then the wells were washed three times with PBS and blocked with 2% (w/v) bovine serum albumin (BSA) in PBS at room temperature for 1 h. Following the removal of BSA, the wells were once more washed three times with PBS, just before the addition of *A. baumannii* strains (100 µl), previously grown overnight at 37 °C in MHB (initial inoculum of approx. 10⁸ CFU ml⁻¹), non-

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supplemented or supplemented with compound **1** at 70 $\mu\text{g ml}^{-1}$, 35 $\mu\text{g ml}^{-1}$, and 10 $\mu\text{g ml}^{-1}$. The plates were subsequently incubated at 37 °C for 3 h, after which non-adherent bacteria were washed out with PBS and the adhered cells collected by adding of sterile PBS supplemented with 0.5% Triton X-100 (125 μl). Serial tenfold dilutions of lysates were then plated onto Luria-Bertani agar (LBA) and incubated at 37 °C for 24 h in order to enumerate the colonies.

Surface Motility Assay

Surface motility was tested on 0.3% agarose plates, containing 2.5 g l^{-1} NaCl and 5 g l^{-1} of tryptone, as described with slight modification.^[65] Cultures in MHB, with an inoculum size of 1.5×10^8 CFU ml^{-1} , treated with 70 $\mu\text{g ml}^{-1}$, 35 $\mu\text{g ml}^{-1}$, and 10 $\mu\text{g ml}^{-1}$ of compound **1**, along with untreated control, were grown at 37 °C to an early stationary phase, after which the freshly prepared plates were point inoculated with bacterial suspensions (2 μl). The plates were subsequently incubated at 37 °C in dark for 42 h i.e. until the appearance of specific motility patterns. ImageJ 1.52a software (National Institutes of Health, Bethesda, MA, USA) was used for the calculation of surface areas covered by migrating bacteria.

Colorimetric Quantification of AHLs

AHL production was screened by colorimetric quantification method.^[66] The strains, inoculated at 1.5×10^8 CFU ml^{-1} , were grown overnight in MHB (5 ml) supplemented with compound **1** at 70 $\mu\text{g ml}^{-1}$, 35 $\mu\text{g ml}^{-1}$, and 10 $\mu\text{g ml}^{-1}$. Then the tubes were centrifuged at 5,000 rpm for 15 min, and the cell pellets were discarded. Supernatants were collected and filtered through a 0.22 μm pore size filters, in order to eliminate cell debris. The filtrates were mixed with ethyl acetate in 2:1 ratio and vortexed for 10 min, after which the mixtures were left to stand for 5 min. The upper organic portions were recovered, and the procedure was repeated twice with the remaining lower aqueous portions. Collected organic portions were then dried at 40 °C, and each sample (160 μl) was transferred into wells of 96-well plates supplemented with 1:1 mixture of 2 M hydroxylamine and 3.5 M NaOH (20 μl), and 1:1 mixture of FeCl_3 (10% in 4 M HCl) and 96% (v/v) ethanol (20 μl). The ODs of dark brown colored ferric hydroxamate complexes were then measured at 520 nm.

Statistical Analysis

All experiments were performed at least three times and the results are presented as mean values \pm SDs. One-way analysis of variance (ANOVA), followed by Tukey's post hoc test was used for comparisons between control and experimental groups. Values for $P < 0.05$ or less were considered to be statistically significant. Statistical analysis was carried out and graphs were prepared by using GraphPad Prism 8 software (San Diego, CA, USA).

Supplementary Material

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/cbdv.202000786>.

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Author Contribution Statement

Dušan Ušjak collected bacterial strains, performed experiments regarding biofilm production, QS activity, motility, and growth curves, assisted in other experiments, analyzed the data, and wrote the article. Miroslav Dinić and Katarina Novović performed Real-Time PCR and adhesion assays. Miroslav Dinić also assisted in study design, data interpretation, and writing of article. Branka Ivković synthesized the compounds. Nenad Filipović and Magdalena Stevanović performed XRD, microscopy, and thermal analyses. Marina T. Milenković designed the study and supervised the work.

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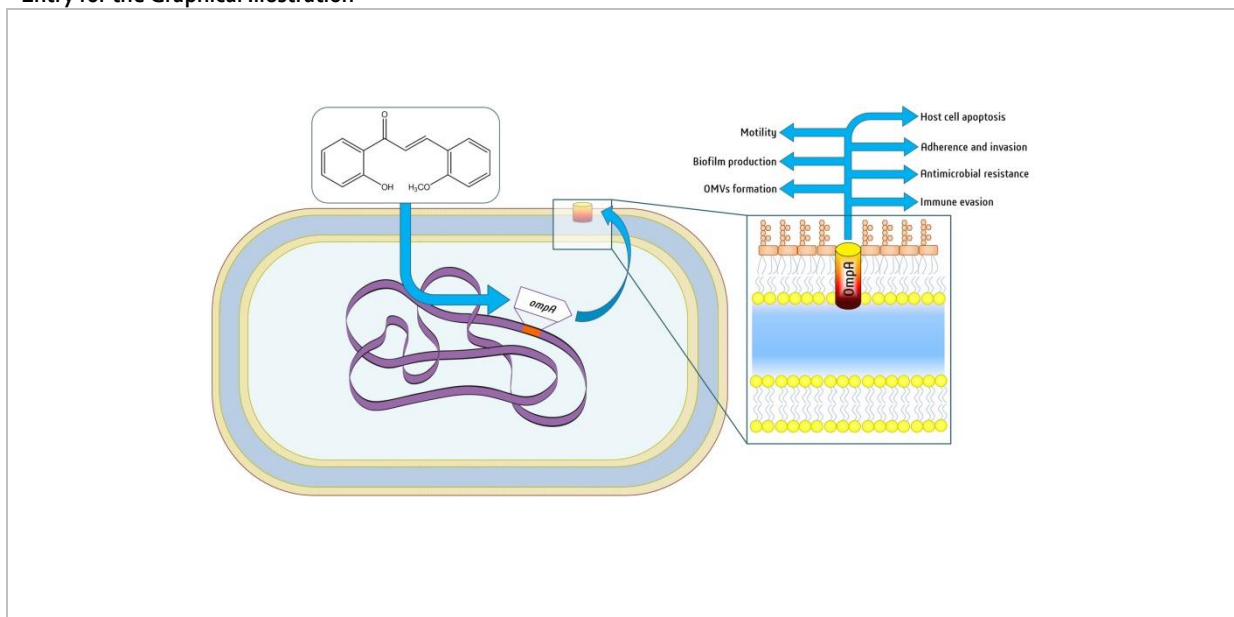
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Entry for the Graphical Illustration



Twitter Text

2-methoxy substituted hydroxychalcone has a potential to prevent serious illness in *A. baumannii* infected patients through downregulation of *bap* and most notably *ompA* gene expression.